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Production of bioactive triterpenes by *Eriobotrya japonica* calli

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Abstract

Callus tissue cultures induced from an axenic leaf of *Eriobotrya japonica* (Rosaceae) produced triterpenes in large amounts (ca. 50 mg/g dry wt). Nine triterpenes were characterized as ursolic acid, oleanolic acid, 2α-hydoxyursolic acid, maslinic acid, tormentic acid, 2α, 19α-dihydroxy-3-oxo-urs-12-en-28-oic acid, hyptadienic acid and a mixture of 3-*O*-*cis*-*p*-coumaroyltormentic acid and 3-*O*-*trans*-*p*-coumaroyltormentic acid. The triterpene composition in the callus tissues was noticeably different from that in intact leaves. The contents of tormentic acid with antidiabetic action, and 2α, 19α-dihydroxy-3-oxo-urs-12-en-28-oic acid with anti-HIV activity, were much larger than those in the intact leaves. All of the triterpenes isolated from the callus tissues showed an inhibitory effect comparable to (−)-epigallocatechin gallate (EGCG) of green tea on the activation of Epstein–Barr virus early antigen (EBV-EA) induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). 2α, 19α-Dihydroxy-3-oxo-urs-12-en-28-oic acid was the most potent inhibitor among them and caused a significant delay of two-stage carcinogenesis on mouse skin.

Keywords: *Eriobotrya japonica*; Rosaceae; Callus tissue culture; Triterpene; Epstein–Barr virus; Two-stage carcinogenesis

1. Introduction

Leaves of *Eriobotrya japonica* Lindl. (Rosaceae) have been used in folk medicine to treat various skin diseases and diabetes mellitus. Triterpenoid constituents have been isolated from the leaves of this plant, and several of them were reported to have anti-tumor, antiviral, and anti-inflammatory properties (Shimizu et al., 1986, 1996; Liang et al., 1990; Tommasi et al., 1992; Nozato et al., 1994). Occurrence of polyphenolic constituents in the leaves and their cytotoxicity against human oral tumor cell lines were also reported (Ito et al., 2000).

We previously reported the production of pharmacologically active compounds by plant cell cultures induced from medicinal plants (Taniguchi et al., 1997, 1998, 2000). In the course of this study, we have established callus cultures of *E. japonica* which produce large amounts of bioactive triterpenes. This paper deals with the characterization of triterpenes produced in the cultured calli, and the difference in the triterpene composition from that in the intact leaves. The anti-tumor properties of these triterpenes are also reported.

2. Results and discussion

2.1. Establishment of callus tissues

Callus tissues of *E. japonica* were induced from an axenic leaf of a seedling on Linsmaier–Skoog (LS) agar medium (Linsmaier and Skoog, 1965) supplemented with 10 μM 1-naphthaleneacetic acid (NAA) and 10 μM 6-benzyladenine (BA), and maintained for over 4 years by subculturing on LS agar medium containing the above plant hormones at 1 monthly intervals at 25 °C in the dark. The calli appear pale yellow and tend to

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aggregate to form clusters. The callus cells grew constantly to reach 5 times the inoculum weight in 6 weeks.

2.2. Characterization of metabolites produced in the callus tissues

Lyophilized callus tissues were extracted with EtOH, and the EtOH extract was partitioned between water and EtOAc. The EtOAc soluble portion was purified by CC on Si gel and ODS gel, and also by preparative HPLC to yield nine triterpenes (**1**–**6**, a mixture of **7**, **8** and **9**). Isolation of the triterpenes was much easier from the callus tissues than from the intact plant, because of the absence of chlorophylls in the callus tissues which were cultured in the dark.

Ursolic acid (**1**) and oleanolic acid (**2**) were identified by comparing their spectral data with those of authentic samples (purchased from TCI and Aldrich).

Compounds **3** and **4** showed ^{1}H and ^{13}C NMR spectroscopic signals assignable to an urs-12-en-oic acid and an olean-12-en-oic acid, and were identified as 2α-hydroxyursolic acid (Numata et al., 1989; Kitajima and Tanaka, 1993), and maslinic acid (Ikuta et al., 1995), respectively, based on comparisons with published data.

Compound **5** showed a $[M+H]^+$ ion peak at *m/z* 489, corresponding to $C_{30}H_{48}O_5$. The ^{1}H NMR spectrum of **5** exhibited signals of an olefinic proton at δ 5.58 (*t*, *J* = 3.5 Hz, H-12), six singlets (each 3H) and a doublet (3H), which were characteristic of the ursene skeleton. The spectrum also showed a singlet at δ 3.04 (*s*, H-18) and two oxygen-bearing methine protons at δ 4.09 (*ddd*, *J* = 4.0, 9.5, 10.0 Hz, H-2β) and δ 3.37 (*d*, *J* = 9.5 Hz, H-3α), suggestive of the 2α, 3β, 19α-trihydroxy structure. The ^{13}C NMR spectrum of **5** substantiated the presence of a pair of olefinic carbons [δ 128.0 (C-12), 140.0 (C-13)], a carboxylic acid group [δ 180.6 (C-28)] and three hydroxylated carbons [δ 68.6 (C-2), 72.7 (C-19), 83.9 (C-3)] on the ursene structure. The identity of **5** as tormentic acid was confirmed by comparison of spectral data (Numata et al., 1989; Kitajima and Tanaka, 1993).

Compound **6** had a ^{1}H NMR spectrum similar to that of **5**, except for the absence of H-3, and a downfield shift and different splitting pattern of the H-2 signal [δ 4.09 (*ddd*) (**5**) → δ 4.88 (*dd*) (**6**)]. The ^{13}C NMR spectrum of **6** showed signals of a ketonic carbon at δ 216.5 (C-3) and two alcoholic carbons [δ 69.8 (C-2), 72.7 (C-19)] in addition to a pair of olefinic carbons [δ 127.6 (C-12), 140.1 (C-13)] and a carboxylic acid group [δ 180.6 (C-28)] with the structure of ursolic acid. Acetylation of **6** in the usual way gave the monoacetate (**6a**). Based on these data, compound **6** was assumed to be 2α, 19α-dihydroxy-3-oxo-urs-12-en-28-oic acid, and its identity was confirmed by comparing its spectral data with those reported for the compound isolated from *Geum japonicum* (Xu et al., 1996).

Compounds **7** and **8** were obtained as an unseparable mixture. The ^{1}H NMR spectrum indicated the presence of *cis* and *trans* coumaroyl moieties as revealed by the respective signals [*cis*-isomer; δ 6.06, 6.89 (1H, each *d*, *J* = 13.0 Hz), δ 7.11, 8.11 (2H, each *d*, *J* = 8.5 Hz) and *trans*-isomer; δ 6.65, 7.98 (1H, each *d*, *J* = 16.0 Hz), δ 7.14, 7.54 (2H, each *d*, *J* = 8.5 Hz)]. Besides these signals, proton signals of the triterpene residue, which forms a pattern similar to that of **5**, were observed. The ^{1}H NMR spectrum also showed H-3 signals at δ 5.23 and 5.17, which were shifted downfield from that of **5**, indicating the presence of *cis* and *trans p*-coumaroyl groups at C-3 of **5**. The assigned structures for **7** and **8** were confirmed by alkaline hydrolysis, yielding *cis* and *trans p*-coumaric acid along with tormentic acid. Compounds **7** and **8** were thus characterized as 3-*O*-*cis*-*p*-coumaroyltormentic acid and 3-*O*-*trans*-*p*-coumaroyltormentic acid, respectively (Numata et al., 1989).

The ^{1}H NMR spectrum of compound **9** showed six singlets (each 3H) and a doublet (3H) due to seven methyl groups, a singlet at δ 3.02 (H-18) and an olefinic proton at δ 5.58 (*t*, *J* = 3.5 Hz), which were indicative of the 19α-hydroxyursene skeleton. The spectrum also showed another olefinic proton at δ 5.72 (s) and hydroxymethyl protons at δ 4.42 and 4.55 (each 1H, *d*, *J* = 15.0 Hz). Acetylation of **9** gave the monoacetate (**9a**), and its ^{1}H and ^{13}C NMR spectra were fully assigned with the aid of ^{1}H detected multiple quantum coherence (HMQC) and ^{1}H detected multiple-bond heteronuclear multiple quantum coherence (HMBC) spectra (Table 1). Based on these spectral data, **9** was assumed to be hyptadienic acid. In fact, the ^{1}H and ^{13}C NMR spectra were consistent with those reported for hyptadienic acid (Isobe et al., 1996) (Fig. 1).

Table 1
^{1}H–^{13}C one bond and long-range correlations observed in the HMQC and HMBC spectra of compound **9a** (in $CDCl_3$)

δ_H (Proton)	δ_C (Correlated carbon)	
	One bond	Long range
0.80 (H-26)	18.5 (C-26)	33.8 (C-7), 41.4, 41.6 (C-8, 14)
0.95 (H-30)	16.1 (C-30)	41.1 (C-20), 26.0 (C-21), 73.1 (C-19)
0.95 (H-24)	21.3 (C-24)	29.6 (C-23), 47.7 (C-4), 62.9 (C-5), 137.1 (C-3)
1.03 (H-23)	29.6 (C-23)	21.3 (C-24), 47.7 (C-4), 62.9 (C-5), 137.1 (C-3)
1.14 (H-25)	18.6 (C-25)	42.8 (C-9), 50.7 (C-10), 62.9 (C-5), 148.7(C-2)
1.21 (H-29)	27.4 (C-29)	41.1 (C-20), 53.2 (C-18), 73.1 (C-19)
1.28 (H-27)	25.3 (C-27)	28.6 (C-15), 41.4, 41.6 (C-8, 14), 138.3 (C-13)
2.09 ($\underline{Me}CO_2$)	21.0 ($\underline{Me}CO_2$)	
2.53 (H-18)	53.2 (C-18)	129.2 (C-12), 138.3 (C-13), 182.0 (C-28)
4.56 (H-1a)	62.8 (C-1)	137.1 (C-3), 178.7 (C-2), 170.9 ($Me\underline{C}O_2$)
4.70 (H-1b)	62.8 (C-1)	137.1 (C-3), 178.7 (C-2), 170.9 ($Me\underline{C}O_2$)
5.33 (H-12)	129.2 (C-12)	
5.42 (H-3)	137.1 (C-3)	42.7 (C-4), 50.7 (C-10), 62.8 (C-1), 148.7 (C-2)

2.3. Comparison of the composition of the triterpenes in callus tissues and intact leaves

As shown in Table 2, the total amount of triterpenes isolated reached ca. 50 mg/g dry wt. However, the composition of the triterpenes in the callus tissues was found to be quite different from that of the intact leaves of the source plant by HPLC analysis. Table 2 shows the content of each triterpene in the callus tissues in comparison with intact leaves. The contents of **1** and **2** (mono-hydroxylated triterpenes) were lower in the callus tissues than in the intact plant, while the contents of the other triterpenes (di- or tri-hyrdroxylated compounds) were higher in the callus tissues. Compounds **5**, **6** and **9** were not detected in the extracts from the intact leaves. It was noted that compound **4**, which was previously isolated from *E. japonica* leaves as an anti-inflammatory compound (Shimizu et al., 1986), was produced in the calli at 7 times the level in the intact leaves.

In a possible biogenetic pathway for the triterpenoids in the *Eriobotrya* calli (Fig. 2), compounds **3**, **5** and **6** are produced by successive hydroxylations followed by oxidation starting from monohydoxylated triterpene **1**. Compound **9** is supposed to be produced from **5** upon the oxidative cleavage of C-2 and C-3 of the A ring, and closure of the ring (Rao et al., 1990).

2.4. Cytotoxicity against human oral tumor cell lines

The most abundant triterpene in the callus tissues, **5** (20 mg/g dry wt), was previously reported as a hypoglycemic constituent of *Poterium ancistroides* (Villar et al., 1986; Ivorra et al., 1988). Compound **6**, another triterpene (11.8 mg/g dry wt) abundant in the calli, is also known as a potent inhibitor of HIV protease, as well as **1** and **4** (Xu et al., 1996).

Previously, we reported that the procyanidin oligomer from intact leaves showed potent cytotoxicity in tumor cell lines [human oral squamous cell carcinoma (HSC-2), human salivary gland tumor (HSG)], in contrast to a negligible or weak toxicity in human normal gingival fibroblasts (HGF) (Ito et al., 2000). In the present study, we evaluated the cytotoxicity of triterpenes isolated from calli in these tumor cell lines. As shown in Table 3, all of the triterpenes exhibited significant levels of activity with 50% cytotoxic concentration (CC_{50}) values of 10–48 μg/ml, except for **2** and **6**. Although they were also cytotoxic in a normal cell line (HGF), only **2** showed a weak selective cytotoxicity against cancer cell lines relative to normal cells.

Table 2
Contents of triterpenes (mg/g dry wt.) in the calli and intact leaves of *Eriobotrya japonica*

Compound	Calli	Leaves
1	7.6	16.0
2	1.3	1.7
3	6.3	4.6
4	5.6	0.8
5	20.0	n.d.[a]
6	11.8	n.d.[a]
7, **8**	2.9	0.2
9	1.2	n.d.[a]

[a] Less than 0.01 mg/g dry wt.

2.5. Inhibitory effect on EBV–EA activation

Since **1** and **2** were reported to have inhibitory effects on mouse skin carcinogenesis induced by dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Tokuda et al., 1986), we investigated the effects of these and the other triterpenes on the activation of Epstein–Barr virus early antigen (EBV–EA)

Table 3
Cytotoxic activity of triterpenes isolated from the callus tissues of *Eriobotrya japonica*[a]

Compound	CC_{50} (μg/ml)		
	HSC-2	HSG	HGF
1	29	48	25
2	130	230	> 500
3	10	12	12
4	21	26	24
5	21	25	24
6	102	148	184
7, **8**	22	30	50
9	18	18	39

[a] Near confluent cells were incubated for 24 h with various concentrations of test samples, and the relative viable cell number (A_{540}) was determined by the MTT method. The A_{540} of control HSC-2, HSG and HGF cells was 1.444, 1.600 and 0.337, respectively.

Table 4
Relative ratio of EBV–EA activation with respect to positive control (100%) in the presence of triterpene from callus tissues of *Eriobotrya japonica*[a]

	Concentration (mol ratio/TPA)[b]			
	1000	500	100	10
1	0±0.3 (70)[c]	28.2±1.3	66.6±1.4	88.3±0.7
2	8.4±0.5 (70)	29.8±1.1	72.5±2.0	93.7±0.5
3	8.5±0.5 (70)	49.0±1.5	79.6±1.8	100.0±0.3
4	10.3±0.4 (70)	25.4±1.3	70.3±1.9	91.2±0.3
5	6.3±0.5 (70)	45.5±1.8	72.9±1.8	100.0±0.4
6	0±0.2 (70)	23.7±1.0	63.4±1.5	85.2±0.7
7, **8**	13.7±0.5 (60)	30.5±1.5	73.8±1.5	100.0±0.3
9	15.2±0.4 (70)	33.9±1.2	72.1±1.7	100.0±0.3

[a] Values represent percentages relative to the positive control value (100%).

[b] TPA concentration was 20 ng/ml (32 pmol/ml).

[c] Values in parentheses are the percentage of viable number of Raji cells.

induced by TPA as preliminary screening tests for the anti-tumor agents. The results are shown in Table 4. Most of the triterpenes except for compounds **3** and **5** exhibited inhibitory effects comparable to or much stronger (more than 65% inhibition) than that of a positive control, (−)-epigallocatechin gallate (main polyphenol of green tea) at a concentration of 500 mol ratio/ TPA (Ito et al., 1999). Compound **6** having a ketone group showed the most potent activity among the compounds tested (76.3% inhibition at 500 mol ratio/TPA). This is the first report of anti-tumor related activity for **6–9**.

2.6. Anti-tumor activity in two-stage mouse skin carcinogenesis

Compound **6**, the most potent inhibitor of the activation of EBV–EA, was then examined for its effect on two-stage mouse skin carcinogenesis induced by nitric oxide (NO) generated from (+)-(*E*)-4-methyl 2[(*E*)-hy-

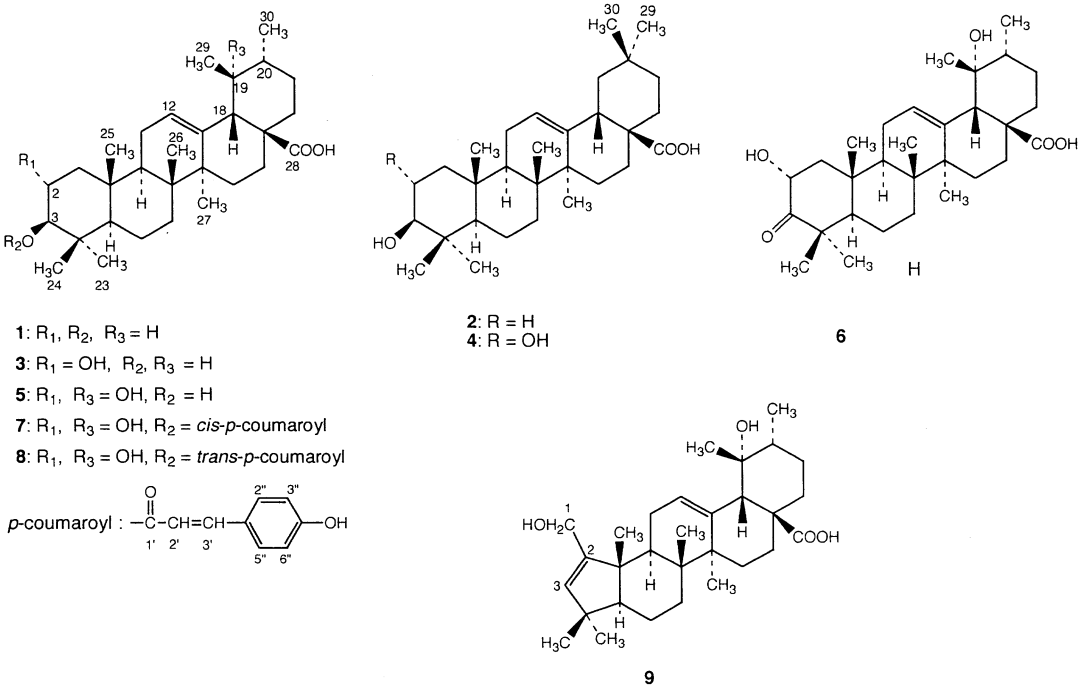

Fig. 1. Compounds isolated from callus tissues of *Eriobotrya japonica*.

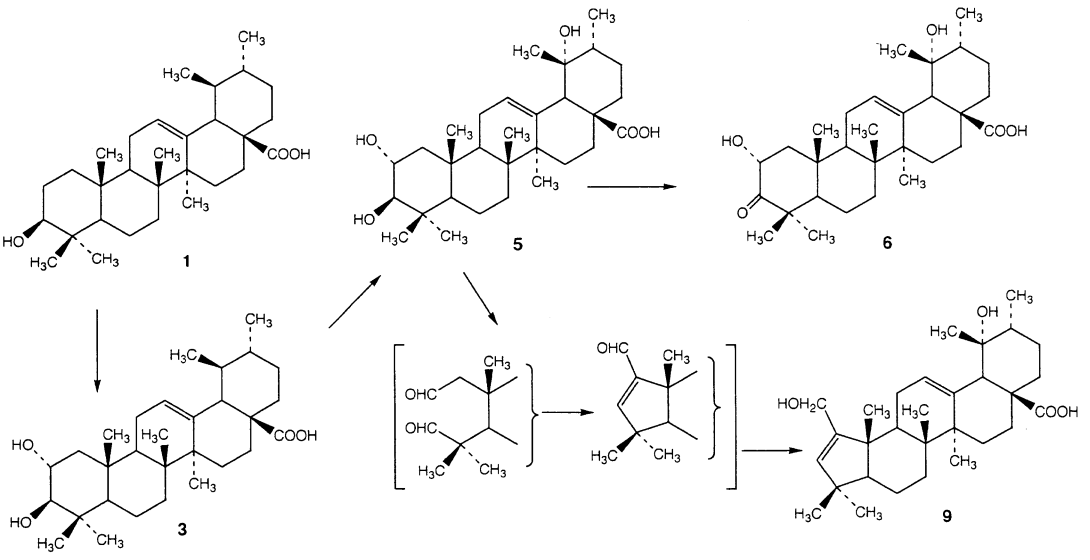

Fig. 2. Possible biogenetic correlation for the triterpenes in the calli of *Eriobotrya japonica*.

droxyimino]-5-nitro-6-methoxy-3-hexenamido (NOR1), (Fukuda et al., 1996) as initiator and TPA as promoter. This assay, which was recently developed by one of the authors (Tokuda) (Takasaki et al., 1999), has the advantage that only a small amount of sample is used (ca. 1/10 the amount for DMBA/TPA-induced carcinogenesis assay). In this assay, EGCG, a well known anti-tumor promoting agent (Yoshizawa et al., 1987), also exhibited a significant anti-tumor initiating activity, although the potency was weaker than that in the DMBA/TPA assay. Compound **6** was found to be an anti-initiating agent comparable to EGCG. The average number of papillomas per mouse upon treatment with **6** also decreased to about 40% of the control value in week 20 (Fig. 3b).

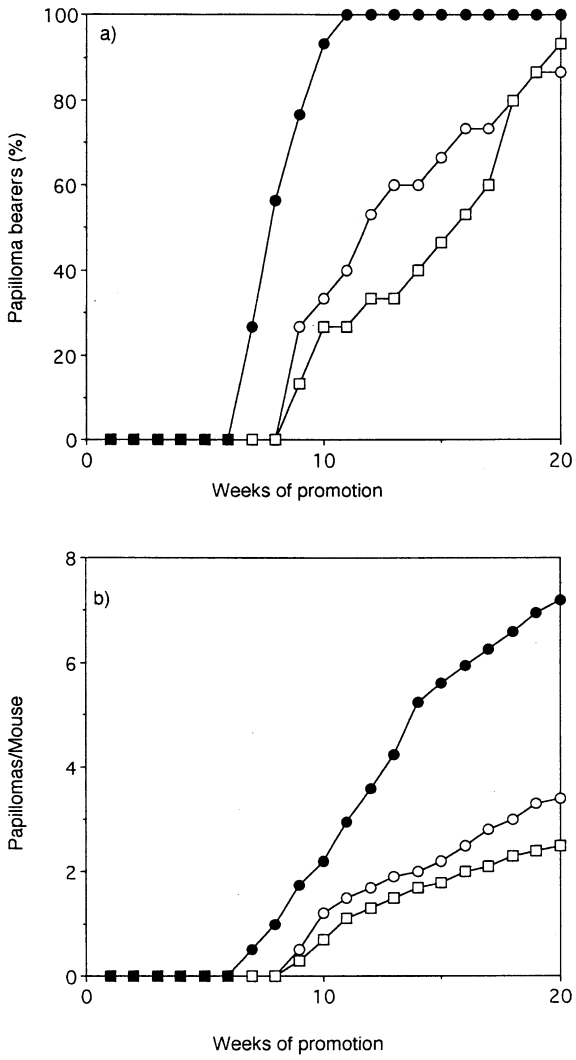

Fig. 3. Inhibitory effects of EGCG and compound **6** on the NOR1-TPA carcinogenesis system. Tumor formation was initiated with NOR1 (390 nmol) and promoted with 1.7 nmol of TPA given twice weekly starting 1 week after initiation. (a) Percentage of mice bearing papillomas. (b) Average number of papillomas per mouse. ● Control (NOR1 and TPA); □ NOR1 + TPA + EGCG (0.0025%); ○ NOR1 + TPA + **6** (0.0025%). Papillomas per mouse of EGCG and **6** treatment were significantly different from control at 20 weeks after promotion, $P < 0.005$.

3. Conclusion

We established cultures of callus tissue of *Eriobotrya japonica* producing triterpenes with biological activity. Among the triterpenes isolated from the tissues, compounds **6**, **7**, **8** and **9** have not been reported as constituents of intact *E. japonica*. The triterpene composition of the callus tissues was noticeably different from that of the leaves of the source plant, the calli producing more hydroxylated and oxidized compounds. The difference in the triterpene composition between callus tissues and intact leaves suggests differences in the expression of the oxidative enzymes. The callus tissues established in this study had the ability to produce preferentially ursolic acid-type triterpenes such as **5** and **6**, rather than oleanolic acid-type triterpenes such as **2** and **4**.

All of the triterpenes showed cytotoxicity in human oral tumor cell lines, except for **2** which had a weak selective cytotoxicity, and **6**. Compound **6** showed a potent inhibition of the activation of EBV–EA induced by TPA, and a significant delay of two-stage carcinogenesis on mouse skin. The *E. japonica* calli is thus regarded as a promising source of biologically active compounds.

4. Experimental

4.1. General procedures

^1H and ^{13}C NMR spectra were measured in pyridine-d_5 or $CDCl_3$ on a Varian VXR-500 instrument (500 MHz for ^1H NMR and 126 MHz for ^{13}C NMR). ESIMS were obtained using a Micromass Auto Spec OA-Tof mass spectrometer with a solvent of 50% MeOH + 0.1% $AcONH_4$. The flow rate was set at 20 μl/min.

4.2. Callus induction and subculture condition

Seedlings were obtained from surface-sterilized seeds (10% antifolmin sol. containing 0.5% Tween 80, 1 h) on sucrose-free LS agar medium. The callus tissues were induced by 1 month-incubation after the inoculation of the segments of seedlings, at 25 °C in the dark. The best formation and growth of calli were observed on LS medium containing 10 μM NAA and 10 μM BA. The callus tissues were subcultured on LS agar medium containing the above plants hormones, at intervals of 1 month over 4 years at 25 °C in the dark.

4.3. Time course of cell growth

Precultured callus tissues (0.4 g) were inoculated to test tubes and cultured in the dark. The tissues were harvested every 7 days with 3 replicates.

4.4. Extraction and isolation

Lyophilized callus tissues (46.6 g dry wt) were extracted with EtOH (2 l×3). The EtOH solution was concentrated in vacuo to give the extract (10.4 g). The extract (9.0 g) was suspended in water (180 ml) and then partitioned with EtOAc (180 ml×7). A portion (5.6 g) of the EtOAc extract (6.7 g) was subjected to CC on Si gel (3.0×20 cm) using a solvent system of $CHCl_3$–MeOH to give four fractions which are rich in triterpenes (Fr. I–IV). Each fraction was purified by ODS CC (Nacalai tesque, cosmosil 75C 18-PREP, 2.2×40–58 cm) with aq. MeOH followed by preparative reversed phase HPLC (YMC, J'sphere ODS M-80, 10×250 mm) to yield **1** (80.1 mg), **2** (6.2 mg), **6** (23.6 mg) and **9** (13.8 mg) from Fr. I (399 mg), **3** (10.8 mg), **4** (11.5 mg) and **9** (5.1 mg) from Fr. II (862 mg), a mixture of compounds **7** and **8** (17.2 mg) from Fr. III (717 mg) and compound **5** (45.1 mg) from Fr. IV (725 mg).

4.5. 2α-Hydroxyursolic acid (3)

Colorless needles, mp 255–258 °C. $[\alpha]_D^{23}$ +48.7° (MeOH; *c* 0.5). ESIMS *m/z*: 473 $[M+H]^+$, 490 $[M+HH_4]^+$. ^{1}H NMR (pyridine-d_5): δ 0.94 (3H, *d*, *J*=6.0 Hz, H-29), 0.97 (3H, *d*, *J*=7.0 Hz, H-30), 0.98 (3H, *s*, H-25), 1.04 (3H, *s*, H-24), 1.07 (3H, *s*, H-26), 1.20 (3H, *s*, H-27), 1.27 (3H, *s*, H-23), 2.62 (1H, *d*, *J*=11.5 Hz, H-18), 3.39 (1H, *d*, *J*=9.0 Hz, H-3α), 4.09 (1H, *ddd*, *J*=4.5, 9.5, 11.5 Hz, H-2β), 5.46 (1H, *t*, *J*=3.5 Hz). ^{13}C NMR (pyridine-d_5): δ 17.0 (C-25), 17.5 (2C, C-26, C-30), 17.7 (C-24), 18.9 (C-6), 21.4 (C-29), 23.8 (C-11), 23.9 (C-27), 24.9 (C-16), 28.7 (C-15), 29.4 (C-23), 31.0 (C-21), 33.5 (C-7), 37.5 (C-22), 38.5 (C-10), 39.4 (C-19), 39.5 (C-20), 39.9 (C-4), 40.1 (C-8), 42.6 (C-14), 48.0 (C-1), 48.1 (C-17), 53.6 (C-18), 55.9 (C-5), 68.6 (C-2), 83.7 (C-3), 125.5 (C-12), 139.3 (C-13), 179.9 (C-28).

4.6. Maslinic acid (4)

Colorless needles, mp 248–250 °C. $[\alpha]_D^{23}$ +59.1° (MeOH; *c* 1.2). ESIMS *m/z*: 473 $[M+H]^+$, 490 $[M+HH_4]^+$. ^{1}H NMR (pyridine-d_5): δ 0.92, 0.97, 0.98, 1.00, 1.06, 1.25, 1.26 (each, 3H, *s*, H-23 to H-30), 3.28 (1H, *dd*, *J*=4.5, 14.5 Hz), 3.38 (1H, *d*, *J*=9.5 Hz, H-3α), 4.09 (1H, *ddd*, *J*=4.5, 9.5, 11.0 Hz, H-2β), 5.45 (1H, *br s*). ^{13}C NMR (pyridine-d_5): δ 16.9 (C-24), 17.5 (C-25), 17.7 (C-26), 18.9 (C-6), 23.7 (C-16), 23.8 (C-30), 24.0 (C-30), 26.2 (C-27), 28.3 (C-15), 29.4 (C-23), 31.0 (C-20), 33.2 (C-7), 33.3 (C-22, C-29), 34.3 (C-21), 38.6 (C-10), 39.8 (C-4), 42.0 (C-19), 42.2 (C-14), 46.7 (C-17), 47.8 (C-1), 48,2 (2C, C-8, C-9), 55.9 (C-5), 68.6 (C-2), 83.8 (C-3), 122.5 (C-12), 144.9 (C-13), 180.2 (C-28).

4.7. Tormentic acid (5)

Colorless needles, mp 272–273 °C. $[\alpha]_D^{23}$ +29.3° (MeOH; *c* 0.3). ESIMS *m/z*: 489 $[M+H]^+$, 506 $[M+NH_4]^+$. ^{1}H NMR (pyridine-d_5): δ 1.00 (3H, *s*, H-25), 1.07 (3H, *s*, H-24), 1.10 (3H, *s*, H-26), 1.11 (3H, *d*, *J*=6.0 Hz, H-30), 1.26 (3H, *s*, H-23), 1.42 (3H, *s*, H-29), 1.70 (3H, *s*, H-27), 3.04 (1H, *s*, H-18), 3.37 (1H, *d*, *J*=9.5 Hz, H-3α), 4.09 (1H, *ddd*, *J*=4.5, 9.5, 10.0 Hz, H-2β), 5.58 (1H, *t*, *J*=3.5 Hz). ^{13}C NMR (pyridine-d_5): δ 16.9 (2C, C-25, C-30), 17.2 (C-26,), 17.7 (C-24), 19.0 (C-6), 24.1 (C-11), 24.7 (C-27), 26.4 (C-16), 27.1 (2C, C-21, C-29), 29.3 (2C, C-15, C-23), 33.5 (C-7), 38.5 (2C, C-10. C-22) 39.9 (C-4), 40.5 (C-8), 42.2 (C-14), 42.4 (C-20), 47.9 (C-9), 48.0 (C-1), 48.3 (C-17), 54.6 (C-18), 56.0 (C-5), 68.6 (C-2), 72.7 (C-19), 83.9 (C-3), 128.0 (C-12), 140.0 (C-13), 180.6 (C-28).

4.8. 2α,19α-Dihydroxy-3-oxo-urs-12-en-28-oic acid (6)

Colorless powder. $[\alpha]_D^{23}$ +22.7° (EtOH; *c* 0.9). ESIMS *m/z*: 487 $[M+H]^+$, 504 $[M+HH_4]^+$. ^{1}H NMR (pyridine-d_5): δ 0.99 (3H, *s*, H-24), 1.09 (3H, *s*, H-26), 1.12 (3H, *d*, *J*=7.0 Hz, H-30), 1.13 (3H, *s*, H-25), 1.21 (3H, *s*, H-23), 1.37 (1H, *d*, *J*=12.5 Hz, H-1α), 1.42 (3H, *s*, H-29), 1.64 (3H, *s*, H-27), 1.87 (1H, *t*, *J*=8.8 Hz, H-9), 2.48 (1H, *dd*, *J*=6.5, 12.5 Hz, H-1β), 3.03 (1H, *s*, H-18), 4.80 (1H, *dd*, *J*=6.5, 12.5 Hz, H-2β), 5.55 (1H, *t*, *J*=3.3 Hz, H-12). ^{13}C NMR (pyridine-d_5): δ 15.9 (C-30), 16.8 (C-26), 17.3 (C-25), 19.6 (C-6), 21.8 (C-24), 24.1 (C-11), 24.7 (C-27), 25.3 (C-23), 26.3 (C-16), 26.9 (C-21), 27.1(C-29), 29. 3 (C-15), 33.2 (C-7), 37.9 (C-10), 38.5 (C-22), 40.4 (C-8), 42.2(C-14), 42.4 (C-20), 47.3 (C-9), 48.1 (C-4), 48.3 (C-17), 50.1 (C-1), 54.6 (C-18), 57.7 (C-5), 69.8 (C-2), 72.7 (C-19), 127.6 (C-12), 140.1 (C-13), 180.6 (C-28), 216.5 (C-3).

4.9. 2α,19α-Dihydroxy-3-oxo-urs-12-en-28-oic acid monoacetate (6a)

Colorless powder. ESIMS *m/z*: 529 $[M+H]^+$, 546 $[M+NH_4]^+$. ^{1}H NMR ($CDCl_3$): δ 0.85 (3H, *s*, H-26), 0.95 (3H, *d*, *J*=7.0 Hz, H-30), 1.12 (3H, *s*, H-24), 1.14 (3H, *s*, H-23), 1.20 (3H, *s*, H-29), 1.24 (3H, *s*, H-27), 1.30 (3H, *s*, H-25), 1.40 (1H, *m*, H-20α), 1.43 (1H, *dd*, *J*=12.5 Hz, H-1α), 1.74 (1H, *dd*, *J*=7.5, 10.5 Hz, H-9), 2.14 (3H, *s*, $\underline{Me}CO_2$), 2.22 (1H, *dd*, *J*=6.5, 12.5 Hz, H-1β), 2.54 (1H, *s*, H-18), 5.35 (1H, *t*, *J*=3.3 Hz, H-12), 5.62 (1H, *dd*, *J*=6.5, 13.0 Hz, H-2β). ^{13}C NMR ($CDCl_3$): 15.9 (C-30), 16.1 (C-25), 17.1 (C-26), 19.1 (C-6), 20.1 ($\underline{Me}CO_2$), 21.3 (C-24), 23.8 (C-11), 24.4 (C-27), 24.8 (C-23), 25.3 (C-16), 25.9 (C-21), 27.4 (C-29), 28.2(C-15), 32.4 (C-7), 37.4 (C-10), 37.9 (C-22), 40.0 (C-8), 41.1 (C-20), 41.3 (C-14), 45.6 (C-1), 46.9 (C-9), 47.7 (C-17), 48.7 (C-4), 52.9 (C-18), 57.0 (C-5), 71.6 (C-2), 73.1 (C-19), 128.5 (C-12), 138.3 (C-13), 170.2 ($Me\underline{C}O_2$), 183.4 (C-28), 209.1 (C-3).

4.10. Mixture of 3-O-cis-p-coumaroyltormentic acid (7) and 3-O-trans-p-coumaroyltormentic acid (8)

Colorless powder. ESIMS *m/z*: 635 $[M+H]^+$. ^{1}H NMR (pyridine-d_5): *cis*-isomer, δ 0.96, (3H, *s*, H-24),

0.97 (3H, *s*, H-25), 1.02 (3H, *s*, H-23), 1.06 (3H, *s*, H-26), 1.10 (3H, *d*, $J=6.0$ Hz, H-30), 1.41 (3H, *s*, H-29), 1.67 (3H, *s*, H-27), 3.03 (1H, *s*, H-18), 4.24 (1H, *td*, $J=4.5$, 10.5 Hz, H-2β), 5.17 (1H, *d*, $J=10.0$ Hz, H-3α), 5.55 (1H, *br s*, H-12), 6.06 (1H, *d*, $J=13.0$ Hz, H-2′), 6.89 (1H, *d*, $J=13.0$ Hz, H-3′), 7.11 (2H, *d*, $J=8.5$ Hz, H-3″, H-5″), 8.11 (2H, *d*, $J=8.5$ Hz, H-2″, H-6″); *trans*- isomer, δ 1.00 (3H, *s*, H-25), 1.02 (3H, *s*, H-24), 1.04 (3H, *s*, H-23), 1.09 (3H, *s*, H-26), 1.12 (3H, *d*, $J=6.5$ Hz, H-30), 1.42 (3H, *s*, H-29), 1.71 (3H, *s*, H-27), 3.05 (1H, *s*, H-18), 4.29 (1H, *td*, $J=3.0$, 10.5 Hz, H-2β), 5.23 (1H, *d*, $J=10.0$ Hz, H-3α), 5.57 (1H, *br s*, H-12), 6.65 (1H, *d*, $J=16.0$ Hz, H-2′), 7.14 (2H, *d*, $J=8.5$ Hz, H-3″, H-5″), 7.54 (2H, *d*, $J=8.5$ Hz, H-2″, H-6″), 7.98 (1H, *d*, $J=16.0$ Hz, H-3′). ^{13}C NMR (pyridine-d_5): *cis*-isomer, δ 16.7, 16.8 (C-25, C-30), 17.2 (C-26), 18.2 (C-24), 18.8 (C-6), 24.1 (C-11), 24.7 (C-27), 26.4 (C-16), 27.0 (C-21), 27.1 (C-29), 29.0 (C-23), 29.3 (C-15), 33.3 (C-7), 38.4 (C-10), 38.5 (C-22), 39.7 (C-4), 40.4 (C-8), 42.2 (C-14), 42.4 (C-20), 47.7 (C-9), 48.3 (C-17), 48.8 (C-1), 54.6 (C-18), 55.6 (C-5), 66.3 (C-2), 72.7 (C-19), 84.9, (C-3), 115.9 (2C, C-3″, C-5″), 116.1 (C-2′), 126.7 (C-2′) 127.8 (C-12), 133.7 (2C, C-2″, C-6″), 140.1 (C-13), 143.5 (C-3′), 160.5 (C-4″), 167.2 (C-1′), 180.8 (C-28); *trans*-isomer, δ 16.8 (2C, C-25, C-30), 17.2 (C-26), 18.2 (C-24), 18.8 (C-6), 24.1 (C-11), 24.7 (C-27), 26.4 (C-16), 27.0 (C-21), 27.1 (C-29), 29.0 (C-23), 29.3 (C-15), 33.3 (C-7), 38.4 (C-10), 38.5 (C-22), 39.8 (C-4), 40.4 (C-8), 42.2 (C-14), 42.4 (C-20), 47.7 (C-9), 48.3 (C-17), 48.6 (C-1), 54.6 (C-18), 55.6 (C-5), 66.4 (C-2), 72.7 (C-19), 85.1 (C-3), 116.8 (2C, C-3″, C-5″), 117.1 (C-2′), 126.3 (C-2′) 127.8 (C-12), 130.6 (2C, C-2″, C-6″), 140.1 (C-13), 144.7 (C-3′), 161.3 (C-4″), 167.9 (C-1′), 180.8 (C-28).

4.11. Alkaline hydrolysis of 7 and **8**

The mixture of **7** and **8** (0.8 mg) was saponificated with methanolic 5% KOH (0.3 ml) for 1.5 h. The reaction mixture was acidified with dil. HCl and then purified with Bond elut C18 (Varian) to give a mixture of *p*-coumaric acid and tormentic acid. The two compounds identified by HPLC with authentic samples [YMC pack ODS-A 303 (4.6×250 mm) column, 0.01 M H_3PO_4–0.01 M KH_2PO_4–CH_3CN = 17:17:6, R_t 11.5 min (*cis*-*p*-coumaric acid), 13.8 min(*trans*-*p*-coumaric acid). YMC pack ODS-A 303 column, 75% MeOH, R_t 14.4 min (tormentic acid)].

4.12. Hyptadienic acid (**9**)

Colorless needles, mp 237–240 °C. $[\alpha]_D^{23}$ +74.5° (MeOH; *c* 1.0). ESIMS *m*/*z*: 471 $[M+H]^+$, 488 $[M+HH_4]^+$. ^{1}H NMR (pyridine-d_5): δ 0.96, 1.06, 1.13, 1.15, 1.39, 1.68 (each 3H, *s*, H-23 to H29), 1.08 (3H, *d*, $J=6.5$ Hz, H-30), 3.02 (1H, *s*, H-18), 4.42, 4.55 (each 1H, *d*, $J=15.0$ Hz, H-1), 5.58 (1H, *t*, $J=3.5$ Hz, H-12), 5.72 (1H, *s*, H-3). ^{13}C NMR (pyridine-d_5): δ 16.7 (C-30), 17.7 (C-6), 18.9 (C-26), 19.0 (C-25), 21.8 (C-24), 25.4 (C-27), 26.4 (C-21), 27.0 (C-11), 27.1 (C-16), 27.2 (C-29), 29.7 (C-15), 30.1 (C-23), 34.6(C-7), 38.5 (C-22), 42.0 (C-4), 42.4 (2C, C-8, C-14), 42.5 (C-9), 43.7 (C-20), 48.3 (C-17), 50.9 (C-10), 54.8 (C-18), 60.8 (C-1), 63.7 (C-5), 72.7 (C-19), 128.2 (C-12), 133.6 (C-3), 140.2 (C-13), 156.8 (C-2), 180.7 (C-28).

4.13. Hyptadienic acid monoacetate (**9a**)

Colorless powder. ESIMS *m*/*z*: 513 $[M+H]^+$, 530 $[M+HH_4]^+$. ^{1}H and ^{13}C NMR data shown in Table 1.

4.14. HPLC Analysis of triterpenes in the callus tissues and intact leaves

Triterpenoids produced in the intact leaves (0.42 g dry wt) and callus tissues (0.30 g dry wt.) were extracted by homogenizing in MeOH (40 ml). After centrifugal separation, an aliquot of the supernatant (2 ml) was evaporated to dryness. The residue (ca. 6 mg) was dissolved in MeOH (4 ml), and after centrifugation, supernatant was injected into the HPLC column (8 μl).

The HPLC analysis was conducted in the following conditions: Condition I, J'sphere ODS-M 80 (4.6×250 mm) column, 78% MeOH, flow rate of 0.8 ml/min; Condition II, YMC-pack ODS-A-303 (4.6×250 mm), 86% MeOH, flow rate of 1.0 ml/min. Detection was effected by UV absorption at 220 nm, and the oven temp. was set at 40 °C. Quantities of the terpenoids were calculated from the peak area by reference to the authentic specimens.

4.15. Assay for cytotoxic activity

HSC-2 and HSG cell lines were maintained as monolayer cultures at 37 °C in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences Lenexa, KS) in a humidified 5% CO_2 atmosphere. HGF cells were isolated from healthy gingival biopsy specimens from a 10-year-old female undergoing periodontal surgery, as described previously (Fujisawa et al., 1999). Cells between the fifth and seventh passages were used.

The relative viable cell number was determined with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, MO). Near confluent cells grown in 96-microwell plates (Falcon, Flat bottom, treated with polystyrene, Becton Dickinson) were incubated for 24 h without or with test compounds. All samples were dissolved in DMSO or 25% DMSO at 50 μg/ml. The final concentration of DMSO in the medium was below 1%. The cells were washed once with phosphate-buffered saline (PBS) and incubated for 4 h with 0.2 mg/ml MTT in DMEM medium supplemented with

10% FBS. After removal of the medium, cells were lysed with 100 μl of DMSO and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate with Labsystems Multiskan® (Biochromatic) and Star/DOT Matrix printer JL-10. The CC_{50} was determined from the dose–response curve.

4.16. EBV–EA activation assay

EBV–EA-positive serum from a patient with nasopharyngeal carcinoma (NPC) was a gift from the Department of Biochemistry, Oita Medical University. The EBV genome-carrying lymphoblastoid cells (Raji cells derived from Burkitt's lymphoma) were cultured in 10% fetal bovine serum (FBS) in RPMI-1640 medium (Nissui). Spontaneous activation of EBV–EA occurred in less than 0.1% of cells in our sub-line. The inhibition of the activation was assayed using Raji cells (virus non-producer type) as described previously (Konoshiama et al., 1989). The indicator cells (Raji, 1×10^6 /ml) were incubated at 37 °C for 48 h in 1 ml of medium containing *n*-butyric acid (4 mmol), TPA (32 pmol, 20 ng in DMSO, 2 μl) as inducer and various amounts of test compounds in 5 μl of DMSO. Smears were made from the cell suspension, and the activated cells that were stained by EBV–EA-positive serum from NPC patients were detected by an indirect immunofluorescence technique (Henle and Henle, 1966). In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) present was recorded. Assays were performed in triplicate for each compound. The average EBV–EA induction of the test compounds was expressed relative to the control experiment (100%) which was carried out in the absence of the test compound. The viability of the treated Raji cells was assayed by the Trypan Blue staining method.

4.17. Two-stage carcinogenesis test on mouse skin

The mice (female ICR, 6 weeks old) were housed, 5 to a polycarbonate cage, in a temperature controlled room at 24±2 °C and given water and food ad libitum throughout the experiment. They were divided into two groups of 15 each.

Mice were topically treated with NOR1 (90 μg, 390 nmol) in 0.1 ml of acetone as an initiating treatment. For the control group, one week after the treatment with NOR1, TPA (1 μg, 1.7 nmol) in 0.1 ml of acetone was topically applied as promoting treatment twice a week and drinking water was provided ad libitum during the experimental period. For the sample-treated group, sample (0.0025%) in drinking water was given from 1 week before to 1 week after the initiating treatment with NOR1, and then mice were treated in the promoting phase in the same way as for the control group.

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